

**Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake.**

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Abbreviations: AVP, arginine vasopressin; CLAMS, Comprehensive Lab Animal Monitoring System; DMH, dorsomedial hypothalamic nucleus; HPA, hypothalamo-pituitary-adrenal; ICV, intracerebroventricular; LC, locus coeruleus; LV, lateral ventricle; NPS, neuropeptide S; NPS-R, NPS receptor; NPY, neuropeptide Y; PVN, paraventricular nucleus.

## **Abstract**

Neuropeptide S (NPS) is a recently discovered peptide shown to be involved in the modulation of arousal and fear responses. It has also been shown that lateral ventricle administration of NPS causes a significant decrease in food intake. Neuropeptides involved in the modulation of arousal have been shown to be involved in the regulation of the hypothalamo-pituitary adrenal (HPA) axis and food intake. In this study we have examined the effect of intracerebroventricular (ICV) administration of NPS on behavior, regulation of the HPA axis and food intake. ICV NPS significantly increased plasma ACTH and corticosterone 10 and 40 minutes post injection respectively. A single ICV injection of NPS caused a significant increase in rearing activity as well as ambulatory movement for up to 45 minutes post injection. We then studied the effect of paraventricular nucleus (PVN) administration of NPS on the regulation of the HPA axis, behavior and food intake. There was a significant increase in plasma ACTH and corticosterone following a single NPS PVN injection. Incubation of hypothalamic explants with increasing concentrations of NPS caused a significant increase in CRH and AVP release. In addition, PVN administration of NPS dose-dependently inhibited food intake in the first hour post-injection although no effect on food intake was seen after this time point. PVN administration of NPS caused a significant increase in rearing activity. These data demonstrate a novel role for NPS in the stimulation of the HPA axis.

## **Introduction**

Neuropeptide S (NPS) is a recently identified 20 amino acid peptide that has been shown to modulate arousal and fear responses. In the rat, the NPS precursor mRNA has been found to be expressed in a large number of tissues with the highest level of expression found in the brain and the thyroid, salivary and mammary glands (1). Within the brain, the highest level of expression is found in the brainstem principally in the locus coeruleus (LC), principle sensory 5 nucleus and the lateral parabrachial nucleus. Low level expression is also found in the dorsomedial hypothalamic nucleus (DMH) and the amygdala. The effects of NPS are mediated via the previously orphan G protein coupled receptor - the NPS receptor (NPS-R). NPS-R mRNA is expressed throughout the CNS with the highest levels of expression found in the cortex, thalamus, hypothalamus and amygdala (1). Intracerebroventricular (ICV) administration of NPS in mice significantly increases locomotion whilst decreasing the amount time spent in slow-wave sleep. In addition, ICV NPS increases exploratory behavior in the open field, light-dark box and elevated plus maze paradigms, models for the study of anxiety-related behavior (1).

A number of hypothalamic neuropeptides involved in the modulation of arousal or anxiety including neuropeptide Y (2), nociceptin (3) and orexin A (4) play a role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis (5-7) and food intake (8;9). It has recently been shown that lateral ventricle (LV) administration of NPS caused a significant reduction in food intake in previously fasted Long Evans rats. The effect on cumulative food intake was seen up to 6 hours post injection. In addition LV administration of NPS significantly inhibited voluntary food intake in rats freely feeding on a palatable diet (10). In these current studies we examine the

effect of ICV NPS on the regulation of the HPA axis, behavior and food intake in male Wistar rats. The paraventricular nucleus (PVN) is an important hypothalamic nucleus involved in the regulation of the HPA axis and food intake. We therefore also examined the effect of intraPVN (iPVN) administration NPS on the HPA axis, behaviour and food intake.

## **Materials and Methods**

### **Materials**

Human NPS was custom synthesized by Bachem (St Helen's, UK). The product was purified to homogeneity by reverse phase high performance liquid chromatography to give >95% purity. Cannulation materials were purchased from Plastic One, Inc. (Roanoke, VA). Reagents for hypothalamic explant experiments were purchased from BDH (Poole, UK).

### **Animals**

Male Wistar rats (specific pathogen free; Charles River, Margate, UK), weighing 250–300 g, were maintained in individual cages under controlled temperature (21–23 C) and light (12-h light, 12-h dark cycle; lights on at 0700 h) with *ad libitum* access to food (RM1 diet, SDS Ltd., Witham, UK) and water. Animal procedures were approved under the British Home Office Animals Scientific Procedures Act 1986 (Project Licence 70/5516).

### **ICV cannulation and injection**

Animals were anesthetized with a mixture of ketamine HCl (60 mg/kg; Ketalar, Parke-Davis, Pontypool, UK) and xylazine (12 mg/kg; Rompun, Bayer Corp., Bury St. Edmunds, UK). Prophylactic antibiotics, flucloxacillin (37.5 mg/kg) and amoxicillin (37.5 mg/kg) were administered prior to surgery. Animals were implanted with a 22-gauge stainless steel guide cannula projecting into the third cerebral ventricle using the co-ordinates calculated from the rat brain atlas of Paxinos and Watson (11) (0.8mm posterior to bregma in the midline and implanted 6.5mm below the outer surface of the skull). Briefly, a Kopf stereotactic frame (David Kopf

Instruments, Tujunga, CA) was used and three stainless steel screws inserted into the cranium. The cannula was fixed to these with dental cement. After surgery, the animals were given 5ml 0.9% saline for circulatory support and buprenorphine (45 µg/kg; Schering-Plough Corp., Welwyn Garden City, UK) for analgesia. The animals were allowed seven days recovery after surgery. They were then accustomed to handling on a daily basis. All compounds were injected using a 28-gauge stainless steel injector placed in and projecting 1 mm below the tip of the cannula. Cannula placement was confirmed by a positive dipsogenic response to angiotensin II (150ng). Only those animals with positive dipsogenic response were included in the data analysis. All animals were habituated to the injection process by a subsequent saline injection.

#### **Intraparaventricular (iPVN) cannulation and injection**

Animals were implanted with a 26-gauge stainless steel guide cannula projecting immediately above the PVN using co-ordinates calculated from the rat brain atlas of Paxinos and Watson (11) (1.8 mm posterior to the bregma, 0.5 mm laterally and implanted 7 mm below the outer surface of the skull) as previously described (12). After a seven day recovery period, animals received two saline injections to habituate them to the injection procedure. All compounds were dissolved in 0.9% saline and administered in a 1µl volume via a 33-gauge stainless steel injector projecting 1mm into the PVN over 1 min. The spread of a 1µl injection into the PVN is reported to be limited to 1mm<sup>3</sup> (13).

### **Study 1a: The effect of ICV NPS on plasma corticosterone and ACTH**

*Ad libitum* fed rats received a single ICV injection of saline, 0.1, 1 or 10nmol NPS (n = 10 per group) in the early light phase (0900–1000 h). Rats were killed by decapitation 10 and 40 minutes post injection. Trunk blood was collected in plastic lithium heparin tubes containing 4200KIU aprotinin (Bayer Corp., Haywards Heath, UK) for corticosterone analysis and in plastic EDTA tubes for ACTH analysis. Plasma was separated by centrifugation, frozen on dry ice, and stored at -20 C until assayed. Plasma TSH and LH were also measured.

### **Study 1b: The effect of ICV NPS on behaviour**

Two behavioral analysis studies were carried out in ICV cannulated animals. In the first study, animals received a single ICV injection of either saline, 1, 3 or 10nmol NPS (n = 8 per group) in the early light phase (0900–1000 h). Following injection, behavioral patterns were monitored continuously for 60 minutes post injection by observers blinded to the experimental treatment. Behavior was classified into eight different categories; feeding, drinking, grooming, burrowing, rearing, locomotion, head down and sleeping adapted from Fray (14). These methods have previously been used to demonstrate abnormal behaviour following CNS administration of peptides (15). During the analysis, each rat was observed for 15 seconds every 5 minutes. This 15 second period was subdivided into three and the behavior of the rat during each time period scored. In the second behavioral study, animals received a single ICV injection of either saline, 0.03, 0.1 or 0.3nmol NPS (n = 8 per group) in the early light phase (0900-1000h). Following injection, behavioral analysis was carried out as described above.

### **Study 1c: The effect of ICV NPS on activity**

ICV cannulated animals were monitored using a 24 chamber open-circuit Oxymax Comprehensive Lab Animal Monitoring System (CLAMS; Columbus instruments, Columbus, OH). Rats were maintained at 24 C under a 12:12hr light-dark cycle (light period 0700-1900). Powdered RM1 diet and water were available *ad libitum* unless otherwise stated. Animals were individually housed in special plexiglass cages, through which air was passed at a flow rate of 2.5L/min.

All rats were acclimatized to their cages for two days and were fasted 24 hours prior to the study. Animals received a single ICV injection of either saline (n = 12) or 10nmol NPS (n = 12) in the early light phase (0900–1000 h). This dose of NPS was chosen because it is similar to the dose given previously which caused a reduction in food intake (10). Animals were returned to their home cage with *ad libitum* access to food. During CLAMS monitoring, the ambulatory activity of each individually housed animal was measured simultaneously using the optical beam technique (Opto M3, Columbus Instruments). Consecutive photo-beam breaks were scored as an ambulatory movement. Cumulative activity counts in x and z axes were recorded every minute for 120 minutes and were used to determine horizontal (XAMB) and rearing (ZTOT) movement respectively.

### **Study 1d: The effect of ICV administration of NPS on food intake**

Groups of rats were fasted for 24 h (n = 10–12) and injected with saline, NPS (0.1, 1, 10 nmol) or 3nmol NDP-MSH in the early light phase (0900–1000 h). This dose of NDP-MSH has previously been shown to inhibit food intake (16). In a separate

experiment, groups of rats were fasted for 24 h (n = 11 per group) and injected with saline, NPS (30nmol) or 5nmol NDP-MSH in the early light phase (0900- 1000 h). Animals were returned to their home cages with a pre-weighed amount of rat chow. Food intake was measured at 1, 2, 4, and 24 hours post injection.

### **Study 2a: The effect of iPVN NPS on plasma corticosterone and ACTH**

Animals received a single iPVN injection of saline, 0.1nmol or 1nmol NPS in the early light phase (0900–1000 h). These doses of NPS were chosen based on previous studies which show that 1/10<sup>th</sup> of the ICV dose is appropriate for intranuclear injection (12). Rats were killed by decapitation 10 and 40 minutes post injection (n = 9 per group per time point). Trunk blood was collected in plastic lithium heparin tubes containing 4200KIU aprotinin for corticosterone analysis and in plastic EDTA tubes for ACTH analysis. Plasma was separated by centrifugation, frozen on dry ice, and stored at -20 C until assayed. Plasma TSH and LH were also measured. In a second study, animals received a single iPVN injection of saline, 0.01nmol or 0.3nmol NPS in the early light phase (0900-1000h). Rats were killed by decapitation and plasma ACTH and corticosterone measured as described above.

### **Study 2b: The effect of iPVN NPS on behavior**

Two studies to examine behavior were carried out on iPVN cannulated animals. In the first study, animals received a single iPVN injection of either saline, 0.1nmol or 1nmol NPS (n = 10 per group) in the early light phase (0900–1000 h). These doses of NPS were chosen based on previous studies which show that 1/10<sup>th</sup> of the ICV dose is appropriate for intranuclear injection (12). In the second study, animals received a

single iPVN injection of either saline, 0.003, 0.01 or 0.03nmol NPS. In both studies, following injection, behavioral analysis was carried out as described above.

### **Study 2c: The effect of iPVN NPS on food intake**

Groups of rats were fasted for 24 h (n = 10–12) and injected with saline, NPS (0.1nmol, 0.3nmol, 1 nmol) or 0.5nmol NDP-MSH in the early light phase (0900–1000 h). This dose of NDP-MSH has previously been shown to inhibit food intake (17). In a separate experiment, groups of rats were fasted for 24 h (n = 11 per group) and injected with saline, NPS (0.003nmol, 0.01nmol and 0.03nmol) or 0.5nmol NDP-MSH in the early light phase (0900- 1000 h). In both studies, the animals were returned to their home cages with a pre-weighed amount of rat chow. Food intake was measured at 1, 2, 4, and 24 hours post injection.

PVN cannula placement was verified at the end of the study by the injection of black ink (18). Data from an animal was excluded if its injection site extended more than 0.2mm outside the PVN.

### **Study 3: Effect of NPS on the release of CRH, AVP and NPY from hypothalamic explants *in vitro*.**

The static incubation system was used as described previously (19). Briefly, *ad libitum*-fed male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with the ventral surface uppermost and placed in a vibrating microtome (Microfield Scientific Ltd., Dartmouth, UK). A 1.8mm slice was taken from the basal hypothalamus and blocked lateral to the circle

of Willis to include the PVN. The hypothalamic slice was incubated in individual chambers containing 1ml artificial cerebrospinal fluid (aCSF; 20mM NaHCO<sub>3</sub>, 126mM NaCl, 0.09mM Na<sub>2</sub>HPO<sub>4</sub>, 6mM KCl, 1.4mM CaCl<sub>2</sub>, 0.09mM MgSO<sub>4</sub>, 5mM glucose, 0.18mg/ml ascorbic acid, and 100µg/ml aprotinin) equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

The tubes were placed on a platform in a water bath maintained at 37 C. After an initial 2 h equilibration period, the hypothalami were incubated for 45 min in 600µl aCSF (basal period), before being challenged with NPS (at doses of 10, 100, and 1000 nM) in 600µl aCSF for 45 min. The viability of the tissue was tested by 45 min exposure to aCSF containing 56 mM KCl. Hypothalamic explants that failed to show peptide release above the basal level in response to aCSF containing 56mM KCl were excluded from the data analysis. Isotonicity was maintained by substituting K<sup>+</sup> for Na<sup>+</sup>. Nine to twelve hypothalamic slices were used for each dose of peptide administered. At the end of each period, aCSF was collected and stored at -20 C until measurement of CRH, arginine vasopressin (AVP) and NPY by radioimmunoassay (RIA).

#### **Study 4: The effect of NPS on ACTH release from pituitary quarters**

The effect of NPS on pituitary ACTH release was determined using anterior pituitary segments. The method was a modification of that previously described (20). Rats were decapitated and anterior pituitary glands were harvested immediately then divided into four pieces of approximately equal size. The segments were randomly placed (one segment per well) into the wells of a 48-well tissue culture plate (Nunc International, Denmark) and incubated in 500µl aCSF. The anterior pituitary

segments were maintained at 37 C in a humidified environment saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 2 h with the medium changed every hour. The segments were then incubated in aCSF alone (control), 100nM NPS, 1000nM NPS or 100nM CRH, a positive control for 4 h (n = 10 per group). At the end of this period, the aCSF was collected and stored at -20 C until RIA for ACTH.

### **Radioimmunoassay**

CRH immunoreactivity (IR), AVP-IR and NPY-IR were measured using established RIA methods (12;21). The intra and interassay coefficients of variation were <10% for the CRH RIA, 11% and 20% for the AVP RIA and <10% for the NPY RIA respectively. Plasma corticosterone was measured using an RIA kit from MP Biomedicals, Inc. (Orangeburg, NY), for which the intra- and interassay coefficients of variation were less than 10% and 7% respectively. Plasma ACTH was measured by immunoradiometric assay purchased from Euro-Diagnostica B.V. (Arnhem, The Netherlands). The intra- and interassay coefficients of variation were both less than 4%. Plasma TSH and LH were measured using in house RIAs using reagents obtained from the National Hormone and Pituitary program (Dr A. Parlow University of California Los Angeles, Harbor Medical Center) methods previously described (22),(23). The intra and interassay coefficients of variation were 8% and 9% for TSH and 9% and 12% for LH respectively. ACTH release from pituitary fragments was measured by RIA using methods and reagents provided by the National Hormone and Pituitary program. The intra and interassay coefficients of variation were 9% and 12% respectively.

## Statistics

Statistical advice was provided by J. Elialoo at the Statistical Advisory Service, Imperial College London. Data for the feeding, hypothalamic explants and chop studies are presented as the mean  $\pm$  SEM. Data for behavioral analysis are presented as median and interquartile range. For both the plasma ACTH and corticosterone studies and feeding studies, groups were compared by one-way ANOVA, followed by *post hoc* Dunnett's test (Systat, Evanston, IL). Data from hypothalamic explant release experiments were analysed using paired Student *t* test between the basal period and the test period. For behavioral studies, data was compared using Kruskal-Wallis One Way ANOVA on ranks. For the CLAMS activity studies differences between the treatment groups were determined using the Mann-Whitney U test (Stata 9, Statacorp, TX). In all cases  $p < 0.05$  was considered to be statistically significant.

## Results

### Study 1a Effect of ICV NPS on plasma ACTH and corticosterone.

ICV administration of NPS caused a significant increase in plasma ACTH 10 minutes post injection compared to saline (plasma ACTH (pg/ml)  $62.6 \pm 9.9$  [saline],  $114.2 \pm 11.0$  [0.1nmol NPS],  $125.0 \pm 8.2$  [1nmol NPS],  $178.4 \pm 19.0$  [10nmol NPS],  $p < 0.05$  0.1nmol vs. saline,  $p < 0.005$  1nmol and 10nmol vs. saline ( $n = 10$ )). There were no significant differences in plasma ACTH by 40 minutes post injection. ICV administration of NPS had no effect on plasma corticosterone at 10 minutes post injection. However, by 40 minutes post injection of NPS there was a significant increase in plasma corticosterone (plasma corticosterone (ng/ml)  $88.8 \pm 32.6$  [saline],  $217.5 \pm 46.3$  [0.1nmol NPS],  $309.3 \pm 25.6$  [1nmol NPS],  $327.6 \pm 52.6$  [10nmol NPS],  $p < 0.05$  0.1nmol NPS vs. saline,  $p < 0.005$  1nmol and 10nmol NPS vs. saline  $n = 10$ )) (Figure 1). No significant changes in plasma TSH or LH were observed at either time point.

### Study 1b: The effect of ICV NPS on behavior

ICV administration of 1nmol NPS caused a significant increase in rearing activity compared with saline treated animals up to 1 hour post injection (median [interquartile range] 4 [2:7] [saline], 26 [22:27] [1nmol NPS]  $p < 0.05$  vs. saline  $n = 8$ ) ICV administration of 3 and 10nmol NPS caused a significant increase in locomotor activity compared with saline treated animals up to 1 hour post injection (4 [2:5] [saline], 6 [3:7] [1nmol NPS], 7 [6:13] [3nmol NPS], 9 [7:10] 10nmol NPS  $p < 0.05$  3nmol and 10nmol NPS vs, saline  $n = 8$ ). NPS also caused a significant reduction in sleeping compared with saline treated controls (20 [13:24] [saline], 0 [0:0] [1nmol NPS], 1.5 [0:6] [3nmol NPS], 0 [0:0] [10nmol NPS]  $p < 0.05$  1nmol and

10nmol NPS vs. saline n = 8. There were no significant changes in any other behaviors (Table 1). ICV administration of lower doses of NPS had no effect on any behaviors compared with saline controls although there was a trend towards a reduction in sleeping (Supplemental Table 1).

### **Study 1c: The effect of ICV NPS on activity**

A single ICV injection of 10nmol NPS caused a significant increase in horizontal movement (XAMB, horizontal beam breaks) and rearing activity (ZTOT, vertical beam breaks). There was a significant increase in horizontal movement from 8 minutes post injection and the effect remained significant up to 44 minutes post injection (Figure 2A). NPS also significantly increase rearing activity between 8 and 34 minutes post injection (Figure 2B). There were no significant differences in activity at any other time points.

### **Study 1d: The effect of ICV NPS on food intake**

ICV administration of NPS 0.1nmol and 1nmol had no effect on food intake. However, 10nmol NPS showed a trend towards an inhibition in food intake 1 hour post injection (0-1 hour food intake (g)  $6.3 \pm 0.6$  [saline],  $6.3 \pm 0.6$  [0.1nmol NPS],  $6.5 \pm 0.4$  [1nmol NPS],  $5.5 \pm 0.5$  [10nmol NPS]). In the same experiment, 3nmol NDP-MSH caused a significant reduction in food intake at one hour post injection (0-1 hour food intake (g)  $6.3 \pm 0.6$  [saline],  $2.9 \pm 0.8$  [3nmol NDP-MSH],  $p < 0.01$  n = 6-11). In a further experiment, 30nmol NPS showed a trend towards an inhibition in food intake (0-1 hour food intake (g)  $6.8 \pm 0.5$  [saline] vs.  $6.1 \pm 0.2$  [30nmol NPS]).

### **Study 2a: Effect of iPVN NPS on plasma ACTH and corticosterone**

NPS significantly increased plasma ACTH 10 minutes post injection (plasma ACTH (pg/ml):  $42.6 \pm 5.1$  [saline],  $106.7 \pm 32.7$  [0.1nmol NPS],  $161.8 \pm 29.5$  [1.0nmol NPS],  $p = 0.09$  0.1nmol NPS vs. saline,  $p < 0.005$  1.0nmol NPS vs. saline ( $n = 9$ )), (plasma ACTH (pg/ml)  $30.4 \pm 5.5$  [saline],  $69.4 \pm 19.0$  [0.01nmol NPS],  $117.3 \pm 11.3$  [0.3nmol NPS],  $p < 0.05$  0.01nmol NPS vs. saline,  $p < 0.005$  0.3nmol NPS vs. saline (Figure 3A + B). iPVN administration of NPS resulted in a significant increase in plasma corticosterone 40 minutes post injection (plasma corticosterone (ng/ml)  $125.1 \pm 35.8$  [saline],  $260.9 \pm 57.7$  [0.1nmol NPS],  $358.4 \pm 72.7$  [1.0nmol NPS],  $p = 0.1$  0.1nmol NPS vs. saline,  $p < 0.01$  1.0nmol NPS vs. saline ( $n = 9$ )), ( $36.3 \pm 12.5$  [saline],  $174.3 \pm 47.4$  [0.01nmol NPS],  $272.4 \pm 34.7$  [0.3nmol NPS],  $p < 0.05$  0.01nmol NPS vs. saline,  $p < 0.005$  0.3nmol NPS vs. saline ( $n = 9$ ) (Figure 3C + D). There were no significant differences in plasma TSH or LH at either time point.

### **Study 2b: The effect of iPVN NPS on behavior**

Intra-PVN administration of both 0.1nmol and 1nmol caused a significant increase in rearing activity up to 1 hour post injection (median [interquartile range] 5 [4:6] [saline], 16 [13:23] [0.1nmol NPS], 14 [9:19] [1.0nmol NPS],  $p < 0.05$  0.1 nmol NPS and 1.0 nmol NPS vs. saline ( $n = 10$  per group)) (Table 2). 1nmol NPS caused a significant decrease in grooming activity (22 [16:24] [saline] vs. 1 [0:1] [1.0nmol NPS],  $p < 0.05$  vs. saline). No other significant differences in behavior were observed between the three groups (Table 2). Intra-PVN administration of lower doses of NPS (0.003, 0.01 and 0.03nmol) showed no differences in behavior compared with saline treated controls (Supplemental table 2).

### **Study 2c: The effect of iPVN NPS on food intake**

NPS significantly reduced food intake in the first hour after iPVN injection in male Wistar rats fasted for 24 hours (0-1 hour food intake (g)  $8.9 \pm 0.5$  [saline],  $7.0 \pm 0.7$  [0.1nmol NPS],  $6.6 \pm 0.3$  [0.3nmol NPS],  $6.0 \pm 0.5$  [1.0nmol NPS]  $p < 0.05$  for all doses vs. saline (n = 10 per group) (Figure 4A)). There were no significant differences in food intake between any of the groups at 2, 4 or 24 hours post injection (data not shown). In a separate experiment, lower doses of NPS (0.03, 0.01 and 0.003nmol) showed no effect on food intake at any time point (Figure 4B).

### **Study 3: Effect of NPS on the release of CRH, AVP and NPY from hypothalamic explants *in vitro***

NPS caused a significant increase in CRH and AVP release from hypothalamic explants. There was no change in NPY release from hypothalamic explants. Actual values are presented in Table 3 and graphically as a % of basal in Figure 5 A-C.

### **Study 4: Effects of NPS on ACTH release from anterior pituitary fragments.**

NPS had no significant effect on ACTH release from pituitary segments. However, there was a significant increase in ACTH release from pituitary incubated in 100nM CRH (positive control) (ACTH release (pg/ml)  $41.0 \pm 5.5$  [control],  $59.5 \pm 8.2$  [100nM NPS],  $62.5 \pm 11.3$  [1000nM NPS],  $115.3 \pm 9.4$  [100nM CRH]  $p = 0.1$  1000nM NPS vs. control,  $p = 0.09$  100nM NPS vs. control,  $p < 0.01$  CRH vs control (n = 10 per group)).

## Discussion

Neuropeptide S is a recently discovered peptide that has been shown to modulate arousal and anxiety related behavior. ICV administration of 0.1 or 1nmol NPS to mice caused a significant increase in locomotor activity. ICV administration of NPS also significantly increased wakefulness and reduced the amount of slow wave sleep (1). A number of peptides involved in the modulation of arousal via the LC also stimulate the HPA axis (24-26). We examined the effect of ICV administration of NPS on the HPA axis. NPS caused a significant stimulation of the HPA axis with an increase in plasma ACTH 10 minutes post injection and plasma corticosterone 40 minutes post injection.

The PVN is rich in CRH and AVP neurons (27) and is important in the control of the HPA axis. Direct injection of NPS into the PVN caused a significant increase in plasma ACTH and corticosterone. Our *in vitro* studies demonstrate that NPS stimulates the release of CRH and AVP from hypothalamic explants. This data therefore suggests that NPS stimulates the HPA axis via the release of CRH and AVP. Neither ICV nor iPVN administration of NPS stimulated the release of either LH or TSH suggesting a direct and specific effect of NPS on the HPA axis. In addition, treatment of pituitary segments with NPS did not alter ACTH release suggesting that NPS does not have a direct effect on the pituitary gland. It is therefore likely that the effects of NPS on the HPA axis are mediated via the hypothalamus through the release of CRH and AVP. NPY plays an important role in the regulation of appetite (28) as well as in the control of arousal and anxiety (2;29). Furthermore it has been shown that exogenous NPY stimulates CRH neurons in the PVN and may contribute to the activation of the HPA axis. It may therefore be hypothesised that the effects of

NPS on both food intake and arousal may be mediated via an NPY pathway. However, Beck *et al* (10) have previously shown that NPS is unable to block NPY stimulated food intake suggested that these peptides may work through different pathways. In agreement with this, we have shown that NPS does not affect the release of NPY from hypothalamic explants.

To further elucidate the role of NPS in arousal we examined the effects of ICV NPS on activity. NPS caused a significant increase in both horizontal movement i.e. movement around the cage and in rearing activity. These effects were rapid (occurring within 10 minutes of injection) but short lived with no significant differences in activity seen by 45 minutes post injection. Formal behavioral analysis adapted from Fray *et al* (14) showed that ICV administration of NPS caused a significant increase in rearing and locomotor activity with a significant reduction in sleeping. Having established a role for ICV NPS in activity and behavior, we examined the effect of iPVN NPS on behavior. Injection of NPS directly into the PVN caused a significant increase in rearing activity. This data is in agreement with a previous study which has shown an increase in exploratory activity in mice following NPS injection (1). The LC in the brainstem plays an important role in the regulation of arousal (30) and in particular in the regulation of the sleep-wake cycle (31). Afferent projections to the LC from a number of hypothalamic nuclei have been described (32). Recently, a monosynaptic pathway between the parvocellular region of the PVN and the LC has been demonstrated (24). Therefore it is possible that the effects of NPS on arousal following both ICV and iPVN administration may be mediated through the LC.

Recently, it has been shown that lateral ventricle administration of 1 or 10 $\mu$ g (approx 0.5 and 5nmol) NPS in previously fasted Long Evans rats caused a significant decrease in food intake (10). A number of neuropeptides including NPY (33), orexin A (7), NMU (34) and galanin (35) which modulate arousal and regulate the HPA axis are also important peptides in the hypothalamic control of food intake. In addition to its roles in the regulation of the sympathetic and parasympathetic nervous systems and pituitary hormone secretion, the PVN is an important nucleus in the regulation of energy homeostasis. Lesioning the PVN results in hyperphagia and weight gain (36) furthermore, injection of a number of orexigenic peptides directly stimulate food intake whilst injection of anorectic peptides inhibits food intake (37). Therefore the effects of ICV and iPVN administration of NPS on food intake were investigated. In our studies, ICV administration of 10 and 30nmol NPS to 24 h fasted male Wistar rats showed a trend towards a reduction in food intake. iPVN injection of NPS significantly inhibited food intake one hour post injection. This effect was short lived, with no significant differences in food intake between the groups after the first hour. The effects on food intake following both ICV and iPVN administration are less potent and of shorter duration than the previous report of the anorectic effects of NPS (10). The reason for this difference in the effect of NPS on food intake in our studies compared to previous studies is not clear. However, it should be noted that the experiments of Beck *et al* were carried out in Long Evans rats which were fasted overnight whilst the current study was carried out in 24 h fasted male Wistar rats. It is therefore possible that there is a strain difference in the food intake response to NPS (10). In addition, the difference in the fasting period between the studies may have altered the sensitivity of the effects of NPS on food intake. It is also possible that the

effects of NPS may differ when administered in the LV as opposed to directly in the third ventricle.

In conclusion we have identified NPS as a novel stimulator of the HPA axis. Our study shows that NPS causes a significant increase in rearing and locomotor activity and stimulates the HPA axis in male Wistar rats at lower doses than are required to inhibit food intake. This may suggest that the effects of NPS on the stimulation of activity, the HPA axis and food intake may be occurring via different circuits. Further work is required to determine the precise mechanism by which NPS mediates these effects.

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## Figure Legends

**Figure 1:** Effect of a single ICV injection of NPS (0.1, 1 or 10nmol) or saline in *ad libitum* fed male rats on plasma ACTH (A) and corticosterone (B) at 10 and 40 minutes post injection. \* $p < 0.05$ , \*\*\* $p < 0.005$  vs. saline (n = 10 per group). Results are mean  $\pm$  sem.

**Figure 2:** Effect of ICV NPS on activity. 24 hour fasted rats received a single ICV injection of saline or 10nmol NPS (n = 12 per group). The ambulatory activity of each individually housed animal was measured simultaneously using the optical beam technique. The effect of NPS on movement along the x axis (horizontal beam breaks (A)) and rearing activity (vertical beam breaks (B)) were determined. \* $p < 0.05$  vs saline. Results are mean  $\pm$  sem.

**Figure 3:** Effect of a single iPVN injection of NPS (0.1 or 1nmol) or saline (A + C) or NPS (0.01 or 0.3nmol) or saline (B + D) in *ad libitum* fed male rats on plasma ACTH (A + B) and corticosterone (C + D) at 10 and 40 minutes post injection. \* $p < 0.05$ , \*\*\* $p < 0.005$  vs. saline (n = 9 per group). Results are mean  $\pm$  sem

**Figure 4:** Effect of iPVN NPS on food intake. 24 hour fasted rats received a single iPVN injection of saline or NPS (0.1, 0.3 or 1nmol) (A) or saline or NPS (0.003, 0.01, 0.03nmol) (B) (n = 9 per group). Food intake was measured 1 hour post injection. \* $p < 0.05$  vs. saline. Results are mean  $\pm$  sem.

**Figure 5:** Effect of NPS (10nM, 100nM, 1000nM) on CRH (A) AVP (B) and NPY release (C) from hypothalamic explants. Data presented as % basal release. \*p< 0.05 vs basal release. Results are mean  $\pm$  sem.

	<b>F</b>	<b>D</b>	<b>G</b>	<b>B</b>	<b>R</b>	<b>L</b>	<b>S</b>	<b>H</b>
<b>Saline</b>	0	0	2	1	4	4	20	3
	[0:0]	[0:0]	[0:3]	[0:2]	[2:7]	[2:5]	[13:24]	[3:7]
<b>1nmol</b>	0	0	1	0	26*	6	0*	2
<b>NPS</b>	[0:1]	[0:0]	[0:3]	[0:0]	[22:27]	[3:7]	[0:0]	[0:6]
<b>3nmol</b>	0	0	3	0	12	7*	1.5	12
<b>NPS</b>	[0:0]	[0:0]	[2:5]	[0:1]	[8:13]	[6:13]	[0:6]	[8:13]
<b>10nmol</b>	0	0	1	1	17	9*	0*	7
<b>NPS</b>	[0:0]	[0:0]	[1:3]	[0:2]	[10:21]	[7:10]	[0:0]	[2:18]

**Table 1:** Effect of a single ICV injection of NPS (1, 3 or 10nmol) or saline in *ad libitum* fed male rats on behavior. Animals were observed for 15 seconds every 5 minutes, this 15 second period was subdivided into three and the behavior of the rat during each time period scored. Data presented as median and interquartile range. F, feeding; D, drinking; G grooming; B burrowing; R rearing; L locomotion; H head down, S sleep.

\*p< 0.05 vs. saline

	<b>F</b>	<b>D</b>	<b>G</b>	<b>B</b>	<b>R</b>	<b>L</b>	<b>S</b>	<b>H</b>
<b>Saline</b>	0 [0:2]	0 [0:2]	22 [16:24]	0 [0:1]	5 [4:6]	4 [4:6]	3 [1:8]	15 [8:17]
<b>0.1nmol NPS</b>	1 [0:3]	0 [0:0]	2 [0:3]	0 [0:1]	16* [13:23]	8 [3:10]	0 [0:0]	7 [2:10]
<b>1nmol NPS</b>	0 [0:0]	0 [0:0]	1* [0:1]	0 [0:3]	14* [9:19]	7 [5:8]	0 [0:0]	11 [2:18]

**Table 2:** Effect of a single iPVN injection of NPS (0.1 or 1nmol) or saline in *ad libitum* fed male rats on behavior. Animals were observed for 15 seconds every 5 minutes, this 15 second period was subdivided into three and the behavior of the rat during each time period scored. Data presented as median and interquartile range. F, feeding; D, drinking; G grooming; B burrowing; R rearing; L locomotion; I inactive. \*p< 0.05 vs. saline

Peptide release	Dose of NPS administered to hypothalamic explants					
	10nM		100nM		1000nM	
	Basal	NPS	Basal	NPS	Basal	NPS
<b>CRH</b>	65.3 ± 23.7	79.7 ± 25.7*	48.2 ± 16.6	67.5 ± 26.4	64.3 ± 20.5	138.0 ± 38.6*
<b>AVP</b>	9.5 ± 2.4	15.3 ± 6.0	10.4 ± 3.1	14.0 ± 4.7*	9.7 ± 2.2	16.8 ± 4.0*
<b>NPY</b>	21.8 ± 4.0	30.2 ± 5.8	33.2 ± 8.1	36.0 ± 6.6	27.8 ± 3.8	32.3 ± 4.9

**Table 3:** Effect of NPS (10, 1000, 1000nM) on the release of CRH, AVP and NPY from hypothalamic explants. CRH, AVP and NPY release are expressed as femptomoles per explant. \*p< 0.05 n = 9-12 per treatment.

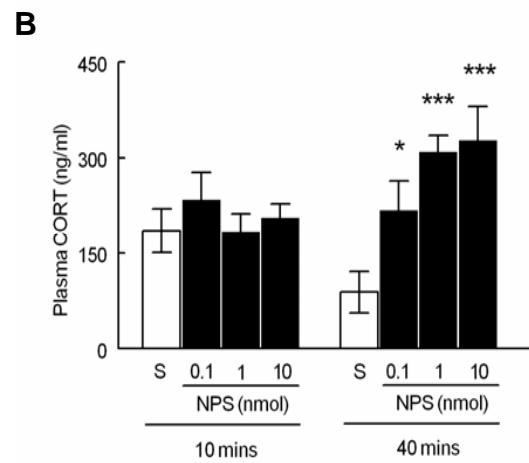
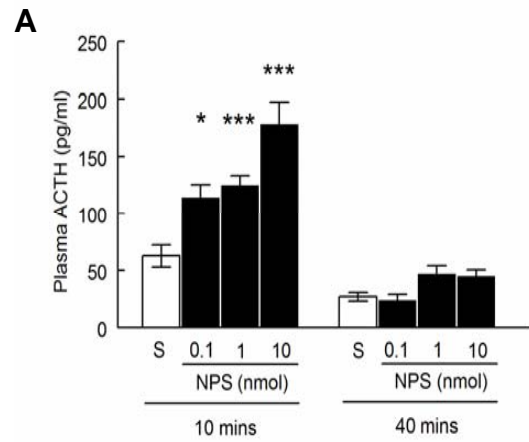


Figure 1

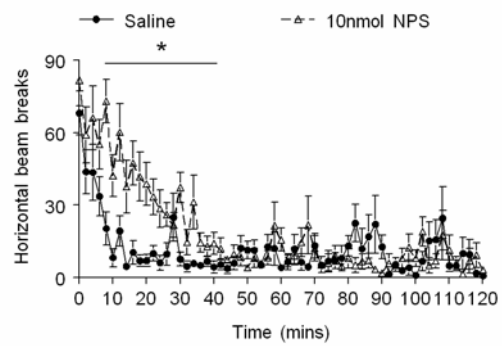
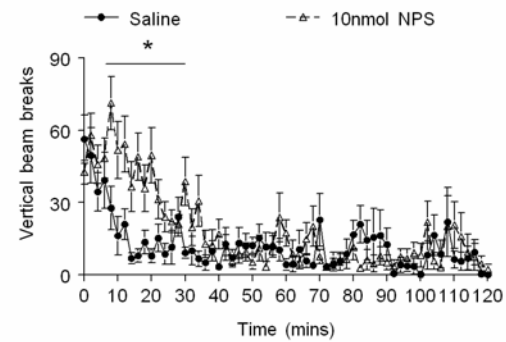
**A****B**

Figure 2

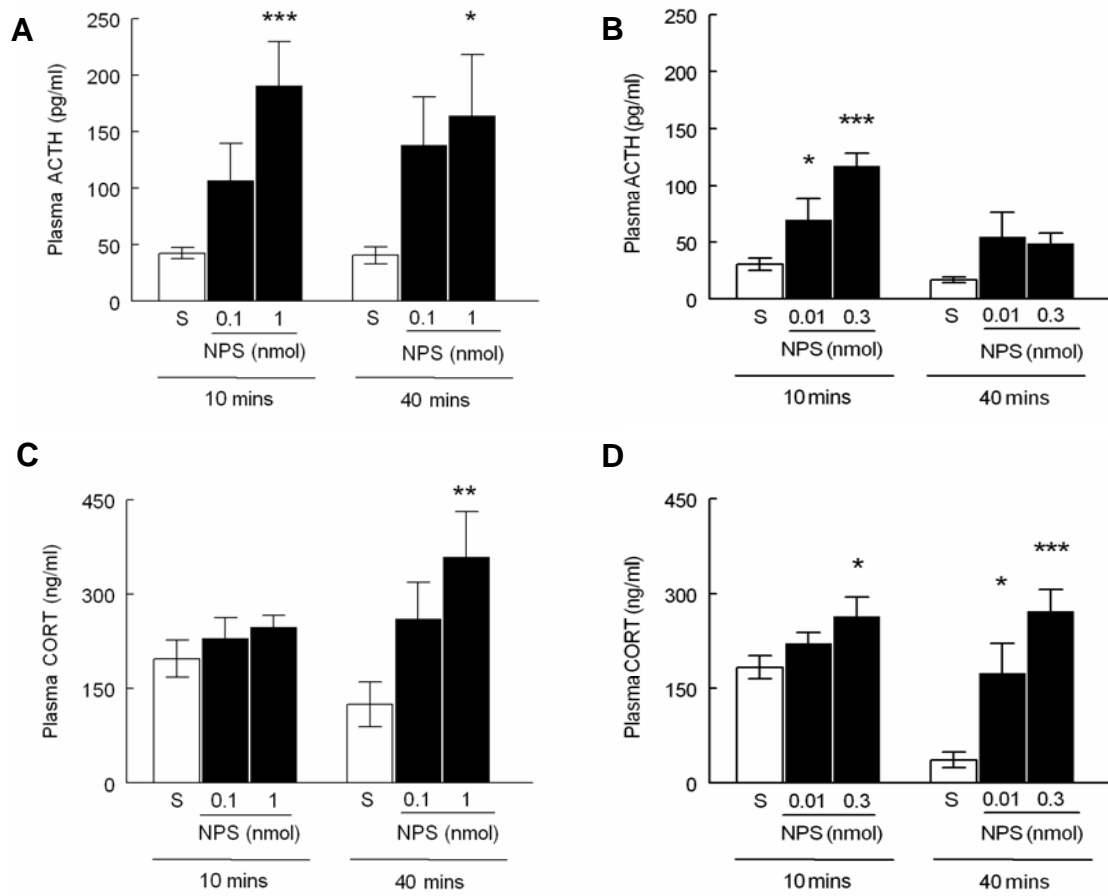


Figure 3

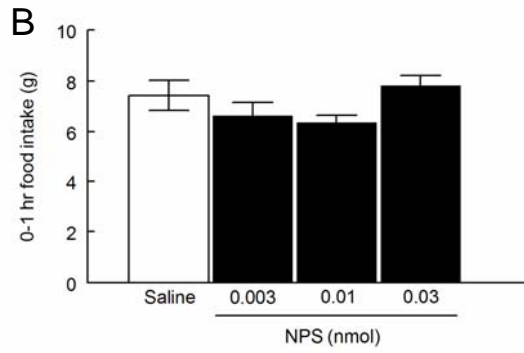
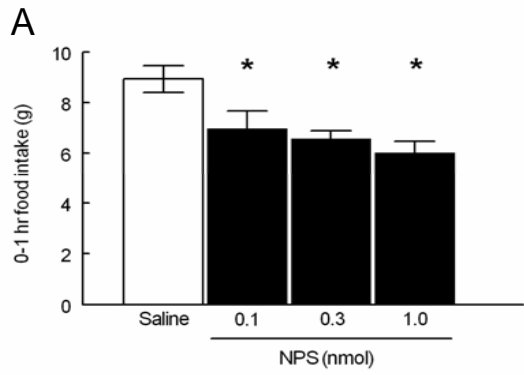


Figure 4

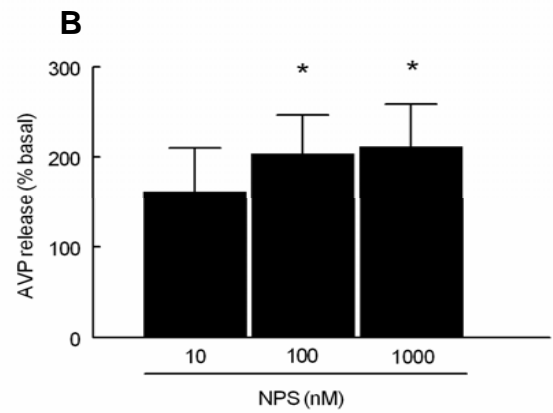
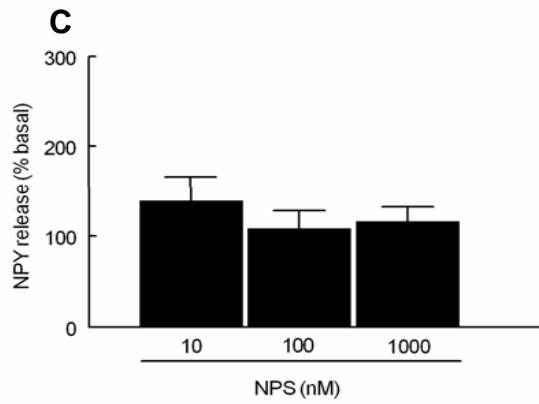
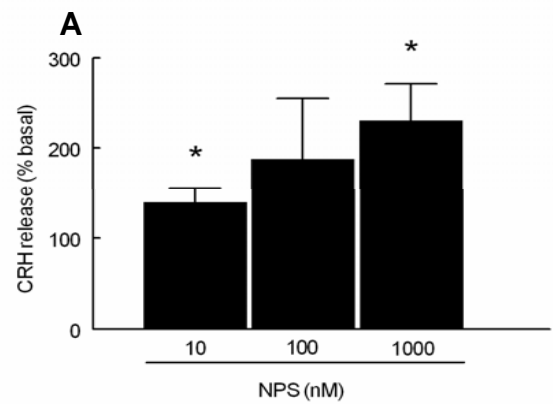


Figure 5